Genetic and Antigenic Typing of Seasonal Influenza Virus Breakthrough Cases from a 2008-2009 Vaccine Efficacy Trial

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Estimations of the effectiveness of vaccines against seasonal influenza virus are guided by comparisons of the antigenicities between influenza virus isolates from clinical breakthrough cases with strains included in a vaccine. This study examined whether the prediction of antigenicity using a sequence analysis of the hemagglutinin (HA) gene-encoded HA1 domain is a simpler alternative to using the conventional hemagglutination inhibition (HI) assay, which requires influenza virus culturing. Specimens were taken from breakthrough cases that occurred in a trivalent influenza virus vaccine efficacy trial involving >43,000 participants during the 2008-2009 season. A total of 498 influenza viruses were successfully subtyped as A(H3N2) (380 viruses), A(H1N1) (29 viruses), B(Yamagata) (23 viruses), and B(Victoria) (66 viruses) from 603 PCR- or culture-confirmed specimens. Unlike the B strains, most A(H3N2) (377 viruses) and all A(H1N1) viruses were classified as homologous to the respective vaccine strains based on their HA1 domain nucleic acid sequence. HI titers relative to the respective vaccine strains and PCR subtyping were determined for 48% (182/380) of A(H3N2) and 86% (25/29) of A(H1N1) viruses. Eighty-four percent of the A(H3N2) and A(H1N1) viruses classified as homologous by sequence were matched to the respective vaccine strains by HI testing. However, these homologous A(H3N2) and A(H1N1) viruses displayed a wide range of relative HI titers. Therefore, although PCR is a sensitive diagnostic method for confirming influenza virus cases, HA1 sequence analysis appeared to be of limited value in accurately predicting antigenicity; hence, it may be inappropriate to classify clinical specimens as homologous or heterologous to the vaccine strain for estimating vaccine efficacy in a prospective clinical trial.

Vaccines based on inactivated or attenuated influenza viruses are an effective strategy to prevent influenza disease, but they rely on an appropriate choice of strains to be used for the vaccine before the season commences (1, 2). The annual selection of vaccine strains in the Northern and Southern hemispheres is necessitated by the continuous antigenic evolution of influenza viruses, which contributes to seasonal differences in the distribution of subtypes and strains as well as the appearance of new subtypes and strains (3–8). Vaccine failure may arise from the emergence of mismatched strains antigenically drifted or unrelated to the vaccine strains (7, 9, 10). Hence, an estimation of the level of vaccine effectiveness may be derived from the determination of the antigenicities of clinical breakthrough strains relative to the relevant vaccine strain.

Genetic changes underlie the emergence of new influenza virus strains (11, 12). Antigenic drift and shift are associated with modifications that include point mutations in the former and reassortment of genetic material between the genomes of viruses coinfecting the same host in the latter (13–15). The evolution of the H3N2 influenza virus strain, since its appearance in humans in 1968, is demonstrative of how drift is also associated with dominant lineage replacement over time (13, 16). Mutations associated with antigenic drift have been identified in the hemagglutinin (HA) gene, including around the sialic acid binding site, as well as other prominent antigenic sites (13, 16–24). The appearance of a new drifted strain is generally associated with several mutations, but drift associated with a single amino acid residue mutation (N145K) may also occur (13).

Although there is a correlation between the antigenic and genetic evolution of influenza virus strains, the predictive power is not necessarily high enough to determine which mutations (even those near the sialic acid binding site) will translate into an antigenically drifted strain (13, 20). Indeed, the same single amino acid substitution in an identical position of the HA1 domain can have opposing effects on phenotype in two different strains of the
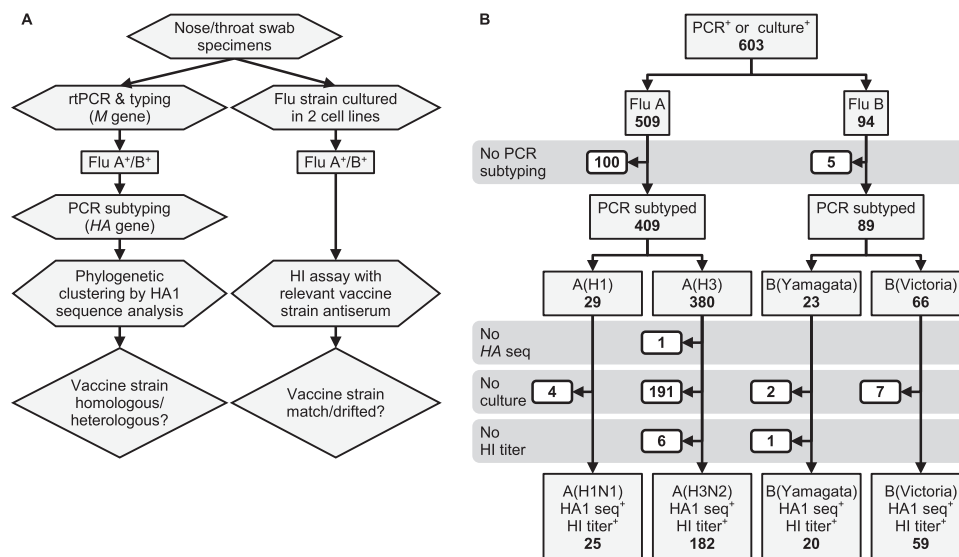
FIG 1 (A) Algorithmic description of influenza specimen characterization from breakthrough case samples. (B) Flow diagram description of the numbers of specimens that were processed in the influenza virus typing, subtyping, and genetic characterization.

same lineage (25). However, genetic-based prediction models of antigenic drift are attractive because they are based on the genetic detection of viruses, and this method is analytically sensitive and relatively easy to perform (7, 15, 26). Yet, genetic-based prediction of antigenicity has not been examined in the context of a prospective vaccine efficacy clinical trial. In these trials, breakthrough cases are relatively infrequent, and the determination of antigenicity has been reliant on the conventional hemagglutination inhibition (HI) assay (27), which is limited by the availability of relevant reference strain ferret antisera (28) and the potential difficulties of cultivating sufficient virus from clinical samples. PCR has already been shown to be a more sensitive technique than culture at detecting influenza virus in nasal/throat swab samples from clinical breakthrough cases after vaccination (29). The aim of this study was to explore the relationship between HA1 domain sequences and antigenicities (determined by HI) of influenza virus strains isolated from clinical breakthrough cases. These cases occurred during the follow up of a large international and multicenter clinical trial evaluating the relative efficacy of two trivalent influenza virus vaccines that was conducted over the 2008-2009 season in >43,000 adults ≥65 years old (30).

(This study has been registered at ClinicalTrials.gov under registration no. NCT00753272.)

MATERIALS AND METHODS

Clinical trial conduct. The observer-blinded randomized trial (ClinicalTrials.gov registration no. NCT00753272 [http://clinicaltrials.gov/show/NCT00753272]) was conducted at multiple sites in 15 countries in the Northern Hemisphere involving subjects who were ≥65 years old at trial entry (30). The trial was approved by the research ethics committees of all participating countries and conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. Written informed consent was obtained from all subjects before trial entry. Approximately half of the subjects received GlaxoSmithKline (GSK) Vaccine's Fluarix (Fluarix is a trade mark of the GlaxoSmithKline group of companies), and the remainder received GSK's candidate formulation of split antigens adjuvanted with AS03$_B$ (GSK Vaccine's proprietary adjuvant system containing 5.93 mg α-tocopherol and squalene in an oil-in-water emulsion). Both

vaccines contained split antigens derived from the strains A/Brisbane/59/2007 (H1N1) (15 μg HA), A/Uruguay/716/2007 (H3N2) (15 μg HA), and B/Brisbane/3/2007 (15 μg HA). A single dose of vaccine was administered intramuscularly in the nondominant arm of each patient.

Sampling. Nasal and throat swabs for culture and PCR were taken up to 5 days after the onset of an influenza-like episode (Fig. 1A) and stored in M4RT transport medium (Remel, United Kingdom) at −70°C. An influenza-like illness was defined as the simultaneous occurrence of at least one respiratory symptom (nasal congestion, sore throat, new or worsening cough, new or worsening dyspnea, new or worsening sputum production, and new or worsening wheezing) and one systemic symptom (headache, fatigue, myalgia, feverishness, and fever [oral temperature of ≥37.5°C]).

Influenza virus culture-based typing. Nose/throat swab samples were stored at −70°C. After thawing, these were cultured both on rhesus monkey kidney (RMK) cells and Madin-Darby canine kidney (MDCK) cells with incubation at 33 to 36°C for up to 2 weeks. Influenza virus A/B typing was performed on fixed cell cultures using standard immunofluorescence histology with influenza virus A-/B-specific antibodies (29).

Influenza virus antigenic typing and HI assay. The HI assay was performed using a standard protocol (31). Validated vaccine strain monospecific antisera were prepared from infected ferrets using a bank of influenza virus vaccine strains (at GlaxoSmithKline Vaccines) and were treated with HA receptor-destroying enzyme (32). The influenza virus antigen controls were produced and validated by the Centers for Disease Control and Prevention. Each HI assay was performed in triplicate using an appropriate vaccine strain virus control or an influenza virus specimen prepared from infected cell cultures (8 HA units/25 μl), serial dilutions of the appropriate ferret-derived vaccine strain antiserum, and 0.5% turkey erythrocytes. The HI titer was defined as the highest dilution step for complete inhibition of hemagglutination. The definition of relative HI titer and the designation of a specimen as vaccine strain matched, drifted, or mismatched are described in Table 1.

Influenza virus detection and PCR typing. Influenza virus detection and A/B typing were performed using quantitative real-time PCR (qPCR) targeting the matrix gene on RNA prepared from total nucleic acid extracted from frozen samples of nose/throat swabs, as described previously (29). Subtyping of influenza virus A-positive cases into seasonal A(H1N1) and A(H3N2) was performed in a separate reverse transcription-PCR (RT-PCR) assay using different sets of primers targeting the hemaggluti-

TABLE 1 Definitions used for the classification of influenza viruses

Entity	Definition
Relative HI titer	A relative HI titer was derived from the ratio of the HI titer for the vaccine-strain antiserum against the virus-specimen (virus specimen HI titer) over the HI titer for the vaccine-strain antiserum against the vaccine-strain antigen (the reference HI titer). A ratio of ≤0.5 was given the value equal to the negative reciprocal of the ratio; a ratio of 1 was given the value of zero, and a ratio of ≥2 kept the same value.
Vaccine-strain matched	Virus with an HI titer that was no more than 4-fold lower than the reference HI titer (i.e., the relative HI titer was ≥−4).
Vaccine-strain mismatched	Virus with an HI titer that was more than 4-fold lower than the reference HI titer (i.e., the relative HI titer was <−4).
Vaccine-strain drifted	Virus that was mismatched and in the same lineage as the vaccine strain
Vaccine-strain homologous	Virus with an HA1 nucleic acid sequence that aligned with the same clade as the vaccine or vaccine-strain homologous reference strain
Vaccine-strain heterologous	Virus with an HA1 nucleic acid that aligned with a clade defined as heterologous to the clade of the vaccine strain

nin (HA) genes for H1 and H3 (30). Classifying influenza virus B-positive cases into B(Yamagata-like) and B(Victoria-like) lineages was done in a separate RT-PCR assay with HA gene-specific primers (30), followed by sequencing (see below).

Sequencing and sequence analysis. The PCR product corresponding to the HA1 domain of the HA gene was sequenced with an automated ABI 3130xl genetic analyzer using a standard DNA sequencing protocol and specific primers. Phylogenetic clustering was performed using the MEGA 4 software with comparisons to vaccine strain homologous or heterologous reference strain sequences as used by the WHO Collaborating Centre for Reference and Research on Influenza, London, United Kingdom (33–35). A virus specimen was classified as homologous to a given vaccine strain when the HA1 nucleic acid sequence of the virus specimen aligned within the same clade as the vaccine strain or vaccine strain homologous reference strain and included the amino acid residue substitution(s) that had also been used to define the clade (33–35). A vaccine specimen was classified as heterologous when its HA1 nucleic acid sequence aligned within the same clade as a vaccine strain heterologous reference strain and included the amino acid residue substitution(s) that had also been used to define the clade (33–35) (see Table 1 for definitions). HA1 sequence group allocation was determined by the amino acid residue substitutions relative to the vaccine strain (or a strain that defined the lineage, in the case of B[Victoria]), using characterized amino acid residue positions in the antigenic sites of the HA1 domain as references (see Table S1 in the supplemental material).

RESULTS

Influenza virus was detected in 603 specimens by qPCR or by cell culture and classified as seasonal influenza A (509 specimens) or influenza B (94 specimens) virus. Further subtyping by PCR classified 380 specimens as A(H3N2), 29 as A(H1N1), 23 as B(Yamagata), and 66 as B(Victoria) (Fig. 1). The pandemic influenza virus A(H1N1)pdm09 was detected in only five specimens by specific qPCR (30) and was not considered for this analysis (not shown). Culturing of the virus was unsuccessful in 50% (191/380) of the A(H3N2) specimens, whereas it was unsuccessful in only 9

to 14% of the specimens from the other subtypes (Fig. 1B). All of the virus specimens that were successfully cultured were also cultured in RMK cells, whereas not all of these specimens were successfully cultured in MDCK cells. Hence, the HI testing was performed with RMK-cultured viruses only. HI titers relative to the relevant vaccine strains and PCR subtyping were determined in 48% (182/380), 86% (25/29), 87% (20/23), and 89% (59/66) of the A(H3N2), A(H1N1), B(Yamagata), and B(Victoria) specimens, respectively.

The subtyped influenza virus specimens for which HA1 sequences were determined were allocated to HA1 domain sequence groups (HA1 groups) based on particular combinations of amino acid substitutions at five antigenic sites (Tables 2, 3, and 4). The 379 A(H3N2) specimens were allocated to 25 groups (Table 2), most of which (259 specimens) were in group 1. The A(H1N1) specimens were allocated to eight groups (Table 3). The 23 B(Yamagata) specimens were allocated to three groups, and the 66 B(Victoria) specimens were allocated to seven groups (Table 4).

An influenza virus was classified as homologous or heterologous to a vaccine strain by phylogenetic clustering based on the HA1 domain nucleic acid sequence, with the vaccine strain and other characterized influenza virus strains as references (Fig. 2, Table 1). A total of 377 A(H3N2) viruses in 24/25 HA1 groups were classified as homologous to the A/Brisbane/10/07 vaccine strain (Fig. 2A). The two A(H3N2) viruses in the remaining group (group 6) were classified as heterologous to the vaccine strain and homologous to the A/Perth/16/09 strain. All 29 A(H1N1) viruses were homologous to the vaccine A/Brisbane/59/07 strain (Fig. 2B). Only two B(Yamagata) viruses from one HA1 group (group 0) were homologous to the B/Florida/04/06 vaccine strain (Fig. 2C). Twenty viruses in the Yamagata lineage were classified as heterologous to the vaccine strain and homologous to the B/Bangladesh/3333/07 strain. Sixty-six viruses in the Victoria lineage were heterologous to the vaccine strain (Fig. 2D). In addition to the RNA mutations associated with the amino acid substitutions that defined the HA1 sequence groups, other mutations were identified, and these contributed to differences in the positioning of individual viruses in the phylogenetic trees.

The A(H3N2) viruses classified as homologous by sequencing included as many as six additional amino acid substitutions in the antigenic sites relative to the vaccine strain (i.e., in HA1 groups 16 and 22), and viruses in the largest HA1 group, group 1, included three additional substitutions (Table 2). The A(H1N1) viruses classified as homologous included as many as four additional amino acid substitutions, and the B(Yamagata) viruses classified as homologous included one additional amino acid substitution (Tables 3 and 4). Nevertheless, the N144K substitution distinguished the two heterologous A(H3N2) viruses from the homologous A(H3N2) viruses, and the S150I/V and N165Y substitutions distinguished the heterologous B/Yamagata viruses from the homologous B/Yamagata viruses (Tables 2 and 4).

Most viruses for which HA1 sequences and relative HI titers were determined belonged to the A(H3N2) lineage (Fig. 1B and Table 5). Relative HI titers were determined for 180 A(H3N2) viruses classified as homologous by sequence, and these titers encompassed a broad range from 2 to −64, with median and mode relative titers of −2 and 0, respectively (Table 5, Fig. 3). In the largest HA1 group, group 1, the 123 relative HI titers also ranged from 2 to −64. Among all the homologous A(H3N2) viruses, 16% (29/180) were classified as drifted (Table 5, Fig. 3). No HA1 group

TABLE 2 Classification of A(H3N2) HA1 domain groups with respect to amino acid substitutions

Strain/HA1 group (n)	Amino acid residue substitutions according to antigenic site with reference to A/Uruguay/716/07 (H3N2)[a]				
	A	B	C	D	E
A/Brisbane/10/07	S138A				
G1 (259)	S138A	P194L		K173Q	
G2 (1)	S138A	L157S, P194L		K173N	K83N
G3 (1)	S138A	P194L		K173R	
G4 (6)	S138A	P194L		K173Q	E62G
G5 (3)	S138A	P194L		K173Q	E62K
G6 (2)	S138A, N144K	P194L		K173Q	E62K
G7 (4)	S138A	P194L		K173Q	G78S/D
G8 (4)	S138A	P194L	S54R/N	K173Q	
G9 (2)	S138A	P194L	E50G	K173Q	
G10 (2)	S138A	P194L	D53N	K173Q	
G11 (4)	S138A	P194L		K173Q	Q57K
G12 (5)	N133N/S, S138A	P194L		K173Q	Y94H
G13 (5)	N122D/I, S138A	P194L		K173Q	
G14 (6)	S138A, R142K	P194L	Q44Q/H	K173Q	
G15 (7)	S138A, N144S	P194L		K173Q	
G16 (7)	S138A	L157S, P194L, A198A/T		K173Q	L59L/I
G17 (6)	S138A	K158R, P194L		K173Q	
G18 (6)	S138A	I192T, P194L		K173Q	
G19 (4)	S138A	P194L		K173Q, V204I	
G20 (6)	S138A	P194L		K173Q, R208I/K	
G21 (4)	S138A	P194L	Q311Q/H	K173Q, I214T/L	
G22 (3)	S138A, M168 M/I	P194L	R299R/K	K173Q	K83K/E
G23 (4)	S138A	P194L		K173Q	R261Q
G24 (8)	S138A	P194L	V309I	K173Q	S262S/N
G25 (20)	S138A, I140I/V/M	D188D/G/N/K, P194L, A196A/T/Y	S45S/N	K173Q	I260I/L

[a] Antigenic site positions and nomenclature in A(H3N2) HA1 domain taken from references 16, 17, 50, 51 and 52.

contained more than one drifted virus apart from HA1 group 1, in which 15% (22/123) were drifted. For the A(H3N2) viruses classified as heterologous by sequence, the two relative HI titers were −4 and −8.

HA1 sequences and relative HI titers were determined for 25 A(H1N1) viruses, and 16% (4/25) of these A(H1N1) viruses were classified as drifted (Fig. 3). The relative HI titers for all viruses tested encompassed a broad range from 2 to −16, with median and mode relative titers of −4 (Fig. 3). In the three HA1 groups with more than three viruses, the relative titers ranged from 0 to −4, −2 to −8, and −2 to −16.

Two viruses classified as homologous by sequence in the B(Y-amagata) lineage gave relative HI titers of −4 for both (Fig. 3).

TABLE 3 Classification of A(H1N1) HA1 domain groups with respect to amino acid residue substitutions

HA1 group (n)	Amino acid residue substitutions according to antigenic site with reference to A/Brisbane/59/07 (H1N1)[a]			
	Ca1	Ca2	Cb	Sb
G0 (1)				K188N, A189T
G1 (13)				A189T, H192R
G2 (5)				A189T
G3 (6)		S141N		A189T
G4 (1)		S141R		A189T
G5 (1)		E140V		A189T, H192R
G6 (1)	E169G		S72P	A189T, H192R
G7 (1)	E169G	S141N		A189T

[a] Antigenic site positions and nomenclature in A(H1N1) HA1 domain taken from reference 53.

Eighteen B(Yamagata) viruses classified as heterologous by sequence gave relative HI titers that ranged from −4 to −32, with median and mode titers of −16. Seventy-two percent (13/18) of these viruses were also classified as drifted. Fifty-nine viruses in the B(Victoria) lineage classified as heterologous by sequence gave relative HI titers that were either −16 or −32, with median and

TABLE 4 Classification of B(Yamagata) and B(Victoria) HA1 domain groups with respect to amino acid residue substitutions

HA1 group (n)	Amino acid residue substitutions according to antigenic site[a]				
	A	B	C	D	E
Yamagata lineage[b]					
G0 (2)			K48R		
G1 (20)	S150I	N165Y	K48R		
G2 (1)	S150V	N165Y	K48R		
Victoria lineage[c]					
G0 (30)	No substitutions				
G1 (22)	No substitutions in antigenic sites				
G2 (4)	I146V			P172S	K75N
G3 (5)	I146V				
G4 (2)	I146V	A202V			
G5 (1)	I146V	K165N		P172S	K75N
G6 (2)		K203T			

[a] Antigenic site positions and nomenclature for influenza B HA1 domain taken from references 54, 55, 56, 57, 58 and 59.
[b] With reference to B/Brisbane/3/07(Yamagata).
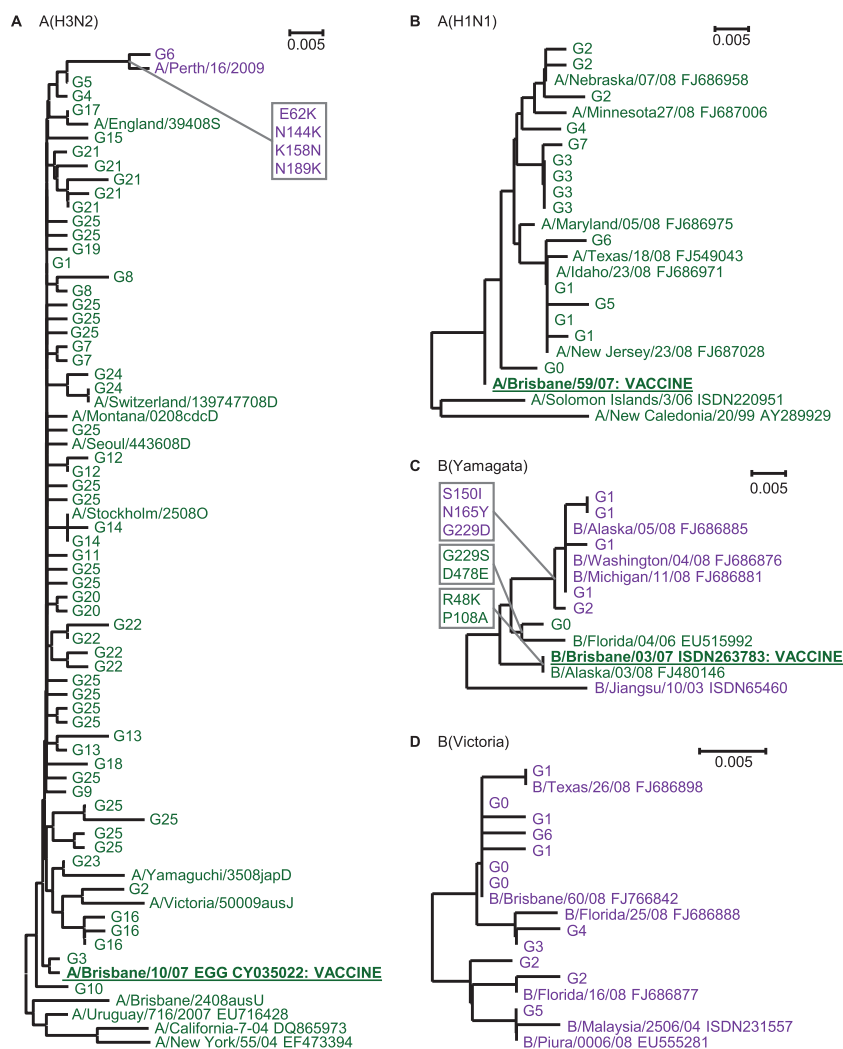[c] With reference to B/Brisbane/60/08(Victoria).

FIG 2 Phylogenetic trees of HA1 domain nucleic acid sequences from influenza viruses calculated with respect to the vaccine strain sequence (in bold, underlined, and dark-green type) and other reference strains (vaccine strain-homologous reference strains also in dark-green type and vaccine strain-heterologous reference strains in dark-purple type) for the four subtypes: A(H3N2) (A), A(H1N1) (B), B(Yamagata) (C), and B(Victoria) (D). (C) Note that B/Alaska/05/08 (GenBank accession no. FJ686885), B/Washington//04/08 (GenBank accession no. FJ686876), and B/Michigan/11/08 (GenBank accession no. FJ686881) are reference influenza virus strains in the B/Bangladesh/3333/07 clade. (A and C) Note that the amino acid residue substitutions that distinguished between clades containing vaccine strain-homologous and vaccine strain-heterologous reference strains are indicated in boxes. The scale bars indicate the fraction of nucleotide substitutions/nucleotide sequence length.

mode titers of −32 (Fig. 3). All these 59 viruses were classified as mismatched.

For each of the influenza viruses classified as homologous by sequence and classified as antigenically drifted, the HA1 domain sequence was determined in the RMK-cultured isolate and compared with the respective sequence determined from the nasal/throat swabs (see Table S2 in the supplemental material). For the 29 A(H3N2) viruses examined after culturing, three (10%) contained revertant (i.e., A138S) or additional amino acid residue substitutions in antigenic sites, and seven (24%) contained amino acid residue substitutions in or next to antigenic sites. For the four influenza A(H1N1) viruses examined after culturing, two (50%) contained additional amino acid residue substitutions in antigenic sites. None of the substitutions affected the designation of a virus as vaccine strain homologous.

DISCUSSION

Influenza virus vaccine effectiveness can differ from one season to the next because of the appearance of strains that are antigenically drifted or mismatched to the vaccine strain (28, 36, 37). Determining whether influenza viruses isolated from clinical breakthrough cases are drifted or mismatched to the vaccine strains is therefore necessary to appropriately estimate vaccine effectiveness (7, 9, 10), but it is challenging because of the requirement to use culture-based methods (26). Moreover, in the context of a vaccine efficacy clinical trial, the number of breakthrough cases detected through culture-based methods may be relatively small, especially if the attack rate is unusually low in a given season (38). The use of PCR is highly attractive because of its sensitivity and ease of application (7, 36, 37, 39). In this study, and in agreement with a recent report, PCR represented a sensitive and accurate method for iden-

TABLE 5 Relative HI titers for A(H3N2) isolates with respect to HA1 domain group

HA1 group by clade/strain	Total no. of sequences	Total no. of HI results	No. of isolates with vaccine-strain relative HI titer of:							
			2	0	−2	−4	−8	−16	−32	−64
Perth/16/09 (heterologous)										
G6	2	2					1	1		
Brisbane/10/07 (homologous)										
G1	259	123	12	37	35	17	13	2	4	3
G25	20	8	1	3	3	1				
G24	8	6			1	2	2	1		
G15	7	6			4	1		1		
G7	4	4	2	1	1					
G18	6	3				1	1			1
G12	5	3	1	2						
G13	5	3		1	1	1				
G16	7	2				1	1			
G14	6	2	1		1					
G20	6	2			1			1		
G8	4	2		2						
G11	4	2	1	1						
G21	4	2			1			1		
G23	4	2			1		1			
G5	3	2			1	1				
G4	6	1			1					
G17	6	1						1		
G19	4	1				1				
G22	3	1					1			
G9	2	1		1						
G10	2	1		1						
G2	1	1			1					
G3	1	1			1					
Total no. (homologous) specimens	377	180	20	58	48	25	18	3	4	4

tifying and typing influenza virus strains in samples from randomized prospective clinical trials (29). PCR also appeared to be more efficient at detecting A(H3N2) viruses than the culture-based methods, possibly related to the reduced sensitivity of culture method with A/Brisbane/10/07 lineage strains (29, 39, 40).

In the current study, the majority of detected strains were from the A(H3N2) lineage (most of which were antigenically matched with the vaccine strain), with a minority of strains being from the seasonal A(H1N1) and B lineages. The relative frequencies of the influenza virus strain subtypes were consistent with the circulating strains observed by the influenza surveillance networks in the countries where subjects were enrolled (e.g., 41, 42). Therefore, certain factors that are common to vaccinated individuals in this study and to those in the general population, such as those related to environmental or genetic predisposition, may have contributed to the occurrence of breakthrough cases. Moreover, breakthrough cases associated with vaccine-matched influenza virus strains were to be expected because seasonal influenza virus vaccines have been found to be only partially effective even against circulating vaccine-matched strains (43).

Using the HA1 nucleic acid sequence to classify influenza viruses as vaccine strain homologous or vaccine strain heterologous was consistent with antigenicity for the majority of viruses examined. Eighty-four percent of the A(H3N2) and A(H1N1) viruses classified as homologous by sequence were matched to the respective vaccine strains, and conversely, 72% of the B(Yamagata) influenza viruses classified as heterologous by sequence drifted from the vaccine strain. Nevertheless, the wide range of titers among the larger HA1 groups suggests that the HA1 domain sequence was not necessarily a reliable predictor of antigenicity or that a particular HA1 substitution was associated with a drift. These wide ranges of titers were most notable in the two largest HA1 groups of A(H3N2) and A(H1N1) viruses classified as homologous by sequence, and corresponded to 128- and 16-fold difference in relative HI titers, respectively. Moreover, the homologous A(H3N2) and A(H1N1) viruses that were classified as antigenically drifted appeared not to be highly associated with particular HA1 groups. And although the HA1 sequence may have harbored other amino acid substitutions not used in the HA1 group classification (data not shown), there was no evidence that these substitutions were associated with drift either.

Other factors may have affected the relative HI titers of the drifted viruses, such as mutations that can potentially reduce virus
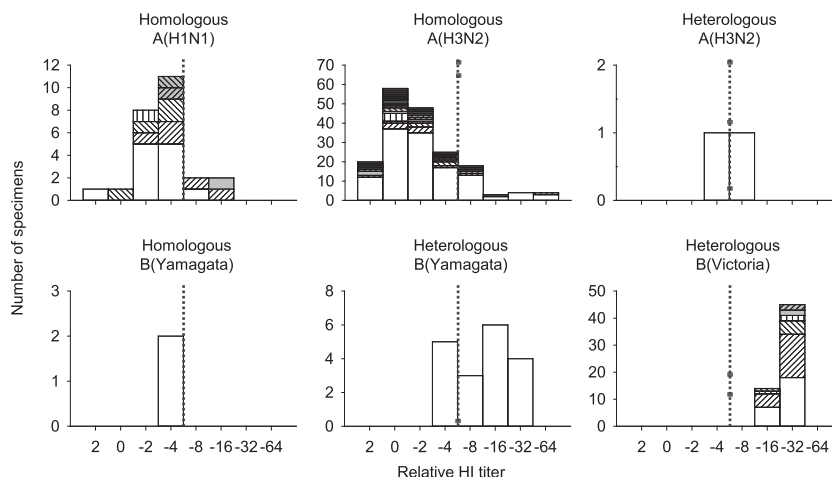

FIG 3 The number of virus specimens according to relative HI titers for homologous and heterologous A(H3N2) types, homologous A(H1N1) types, homologous and heterologous B(Yamagata) types, and heterologous B(Victoria) types. Each bar for a relative HI titer includes the respective number of specimens from each HA1 domain group (separated by horizontal lines in the bar, ranked by the overall total number of specimens in the HA1 group and differently shaded and patterned for the highest eight ranked HA1 groups, with HA1 groups ranked ≥9 in dark gray). Note that no heterologous A(H1N1) specimens were identified, and only single groups of two specimens were identified for heterologous A(H3N2) specimens and homologous B(Yamagata) specimens.

avidity to turkey erythrocytes (44, 45) or mutations that can affect neuraminidase function (45, 46), thus confounding the use of the HA1 sequence alone for classifying vaccine strain relatedness. The cell culture-related amino acid residue substitutions in the HA1 domain also might have affected the HI titers; and although these substitutions were not evaluated further, they were only identified in a minority of the viruses that were classified as homologous by sequence and classified as antigenically drifted. Moreover, such artifacts associated with the cell culturing of virus reflect a potential limitation of the HI assay for antigenic typing (47–49).

Drifted strains with a distinct HA1 group identity may not have been sufficiently prevalent during the 2008-2009 surveillance period to be identified in this study. Similarly, in an influenza virus surveillance study covering the 2009-2010 season in Canada (28), all 60 H3N2 viruses that were classified antigenically were A/Perth/16/2009-like and vaccine homologous, even though the majority of A(H3N2) viruses genetically aligned with A/Hong-Kong/2121/2010, which differs from A/Perth/16/2009 by eight amino acid residue substitutions across the HA1 antigenic sites. Indeed, the time taken for the emergence of a new immunodominant drifted strain was 3.3 years on average in the cluster analysis of H3N2 strain evolution using the HA1 domain sequence (13). Moreover, the center of a new drifted strain cluster was separated from the center of the parental strain cluster by an average of 4.45 antigenic distance units, corresponding to a 22-fold ($2^{4.45}$) difference in relative HI titers, and by an average of 13 amino residue substitutions (13). In the current study, although a wide variation in relative HI titers (and hence in antigenic distances) for a given HA1 group was identified, the genetic variation observed might be accommodated within a single-strain cluster. Hence, in a single season, the HA1 sequence appears to be unsuitable for an estimation of vaccine efficacy or for the identification of potentially new immunodominant strains, because the prediction of antigenicity and class-matched and -mismatched viruses from individual clinical breakthrough cases was not reliable. Therefore, the HI assay should remain the preferred method for determining the relatedness between circulating strains and vaccine strains. However, epidemiological monitoring of genetic evolution performed over numerous seasons, rather than a single season, may provide a basis for more accurate predictions.

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